R81-70

Denaturation of β-Lactoglobulin by Shaking and Its Subsequent Renaturation

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Received January 30, 1981; accepted March 2, 1981

The denaturation of β-lactoglobulin by shaking resembles the inactivation of cellulase under similar conditions. The denaturation is greatly affected by rate of agitation, and by pH; but temperature (30–50°C) has little effect. The rate of denaturation can be reduced to very low levels by the addition of small amounts of surfactants, or of large polymers like polyethylene glycol or methyl cellulose. A high percentage of the shake-denatured β-lactoglobulin can be renatured by dissolving it in dilute acid. Heat denaturation, on the other hand, is not affected by surfactants; and the product formed could not be renatured.

The stabilization of proteins is a broad subject (1, 2). It deals with the factors responsible for modifying molecular structure: heat, chemicals (urea, pH, metal ions), physical forces (shear, shaking, sonication), and biological forces (enzymes, microorganisms). Instability is usually interpreted as denaturation, an unfolding of the protein with expansion in volume but involving no changes in covalent structure. Denaturation is often followed by aggregation and precipitation. The changes in conformation have been followed by a wide variety of techniques. Kauzmann listed 22 of these over 20 years ago (1) and the number continues to increase. The driving force behind all this is the need to develop means (3–5) for increasing stability, to preserve foods, to prolong enzyme life—in todays terms—to conserve.

In this report, we examine the stabilization of one protein, β-lactoglobulin, to one denaturant, shaking, following only changes in solubility and in digestibility, and attempting to enhance stabilization only through the addition of chemical stabilizing agents (3). Such a limited approach is not intended to greatly change current concepts of denaturation, but the results may lead to more effective means for its control.

In previous work (6–9) we have shown that the Avicelase (cellulase) of *Trichoderma reesei* is susceptible to inactivation by shaking, and that this type of inactivation can be prevented by the addition of small amounts of surfactants or related materials. Since the work was done using crude cellulase, we felt that the data should be confirmed using a protein of known properties. Of those available, β-lactoglobulin was selected because it was found to be readily denatured by shaking (10), and was on hand in sufficient amounts for the work to be done. This protein has been the subject of numerous investigations (11–13), so that its structure is well known. Ovalbumin is also suitable for experiments of this type (10), but the protein most thoroughly investigated for shake-denaturation is hemoglobin (14, 15).

METHODS

β-Lactoglobulin (βLG) prepared in these laboratories some years ago was used in

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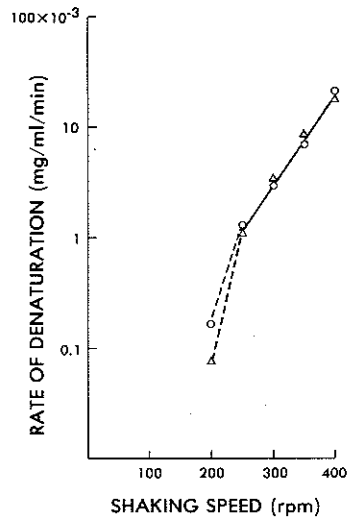

FIG. 1. Effect of rate of shaking, and of temperature on denaturation of β-lactoglobulin. *Conditions*: Protein 1 mg/ml in 0.025 *M* citrate buffer, pH 5.0. ○ —— ○, 50°C; △ —— △, 30°C.

most of the experiments. A commercial sample (cryst: Sigma Chemical Company) was used to confirm the results. Both gave similar gel filtration (HPLC) patterns. β-Lactoglobulin has a p*I* of about 5.2, a molecular weight as the dimer of about 36,000 (11), and an extinction coefficient, *E* 1% 1 cm 280 of 9.5. It is soluble under the conditions of the tests.

The denaturation rate was measured by determining the amount of precipitation as a function of time. The β-lactoglobulin solution (1 mg/ml 0.025 *M* citrate buffer, pH 5.0) was incubated on a variable speed rotary shaker (20 ml/125 ml flask). Samples were removed at appropriate intervals—usually over a 2-hr period—and centrifuged. The protein content of the clear supernatant was determined from the optical density at 280 nm. Where additives interfered with the OD measurements, the amount of protein was determined by redissolving the precipitate in 0.1 *N* NaOH and reading at 280 nm.

The best procedure for renaturation was to dissolve the denatured protein in 0.01 *M* HCl (pH 2.0). At concentrations of protein above 2 mg/ml, resolution was hastened by incubating at 50°C for a few minutes. These solutions could then be readjusted to pH 4.6 by the addition of one-ninth volume of 0.5 *M* citrate buffer pH 5.0, with retention of solubility.

Proteolysis was achieved by incubating 1 ml of protein solution (or suspension) with 0.5 ml of protease (pepsin, mutanolysin) at 50°C pH 4.9. To the digest was added 3 volumes ethanol to precipitate unreacted protein. After 1 hr in the cold, these tubes were centrifuged and the precipitates dissolved in 0.1 *N* NaOH. The change in OD 280 was used as a measure of proteolysis. Mutanolysin was a *Streptomyces*-derived enzyme received from T. Marumo of Dainippon (Japan); the pepsin (3× cryst.) was from Nutritional Biochemical Company.

RESULTS

A. Factors Affecting the Denaturation of β-Lactoglobulin by Shaking

The following factors were investigated for their effects on the denaturation of β-lactoglobulin.

1. Rate of shaking (Fig. 1). The rate of denaturation increases approximately exponentially.

2. Temperature (Fig. 1). At 250 rpm, denaturation was higher at 50 than at 30°C, but at high rates of shaking the denaturation is independent of temperature. This temperature effect may be a function of the time the samples had to be exposed in order to observe denaturation. At low rates of shaking, the incubation period was 8–24 hr; at 400 rpm the incubation was reduced to 15 min.

3. pH (Fig. 2). Denaturation by shaking is greatest at about pH 4.6, with almost none at pH 3.5 and 6.5. The p*I* of β-lactoglobulin is about 5.2 and it is just below this value that denaturation is most rapid.

4. Concentration of β-lactoglobulin (Fig. 3). The apparent half-life of β-lactoglobulin increased 10-fold as its concentration in-

creased over the range (0.5–8.0) investigated (Fig. 3). The initial rate of denaturation (in mg/ml/min) varied by less than a factor of two (Table I). Avicelase inactivation behaved in a similar manner (6). Thus it appears that shaking inactivation is not a first-order reaction as previously assumed (9). First order requires that the half-life be independent of substrate concentration, and that the initial rate be directly proportional to the protein concentration.

5. Miscellaneous factors. The surface–volume ratio affects the rate of denaturation. A fourfold increase in the surface–volume ratio gave about a 10-fold increase in rate. There was an effect of container shape; denaturation was more rapid in a beaker than in a flask of the same diameter. This is a reflection of the increased air–liquid interface in the beaker. Denaturation was not affected by the atmosphere above the solution in the flask (nitrogen vs air), nor by the material of which the container was made (e.g., glass, polyethylene, polypropylene, polycarbonate, stainless steel).

Most experiments were carried out at a shaker speed of 350 rpm and at a temperature of 30°C. Under the standard conditions, over 90% of the β-lactoglobulin was precipitated in 2 hr.

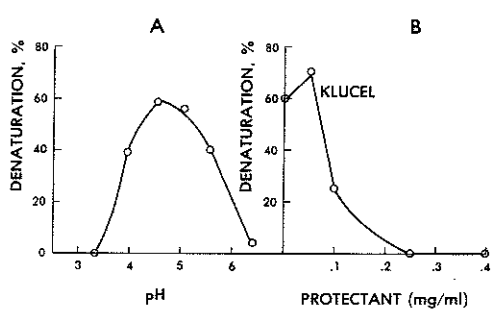

FIG. 2. Denaturation of β-lactoglobulin by shaking, i.e., percentage of total protein precipitated. (A) Effect of pH. Protein 1 mg/ml 0.025 *M* citrate buffer; 400 rpm, 30°C, 45 min. (B) Effect of Klucel E. Protein 1 mg/ml 0.025 *M* citrate buffer containing Klucel at various concentrations, 350 rpm, 50°C, 45 min, pH 5.0.

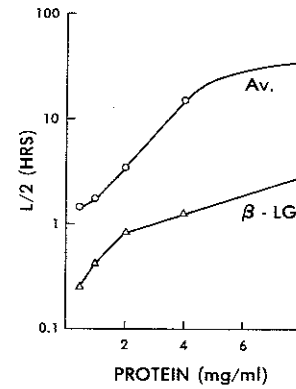

FIG. 3. Effect of protein concentration on stability during shaking (half-life (L/2)). ○ —— ○, Avicelase of *T. reesei* 50°C, pH 5.0, 370 rpm. △ —— △, β-Lactoglobulin 31°C, pH 4.7, 350 rpm.

B. Protection of β-Lactoglobulin against Denaturation by Shaking

A variety of compounds was added to the protein solution in an attempt to minimize the denaturation due to shaking. Some compounds were protective at very low concentrations, the rate of denaturation rapidly decreasing as the concentration increased (Fig. 4). Others, like Klucel E (hydroxypropyl cellulose), increased the rate at very low concentrations, but decreased it greatly at higher concentrations (Fig. 2B). At protective levels, the effect of Zonyl N (0.1 mg/ml) or of digitonin (0.2 mg/ml) was to reduce the rate of inactivation by a factor of about 200.

The protective compounds (Table II) are either simple surfactants usually nonionic, anionic, or amphoteric, or large polymers with surfactant properties to some degree. Zonyl N (DuPont), PEG 6000 (Union Carbide), methocel (Dow), and Tween 80 (Hercules) are the most protective of β-lactoglobulin, being effective at about two parts per 100 parts of protein. It should be noted that while the same compounds protect Avicelase (Table II), their relative effectiveness as protectants for the two proteins may be quite different (compare PPG 2700 and Tween 80). The protection is

TABLE I

Effect of β-Lactoglobulin Concentration on Rate of Denaturation (Precipitation) by Shaking[a]

β-Lactoglobulin (mg/ml)	Rate of Denaturation (ΔOD 280/ml/min)
8	0.021
6	0.019
4	0.016
1	0.014
0.5	0.012

[a] *Conditions*: 0.025 *M* citrate pH 5.0, 350 rpm, 30°C, 20 min. Precipitate taken up in 0.1 *N* NaOH, and OD measured at 280 nm.

independent of temperature (30–50°C), and of pH (tested only with PEG 6000).

Little has been done relating structure to protectant activity. One series of betaine derivatives (Zwittergents) was available in which the alkyl chain varied in length. Those compounds having chain lengths of 14–16 C atoms were as effective as Zonyl N; those of shorter lengths (8–10 C atoms) were much less effective. The relationship is similar to that reported for their protective action on Avicelase (6, 7).

On the other hand, some compounds speed up the denaturation of β-lactoglobulin. Thymol and mineral oil are in this category. One part by weight of thymol per 10 parts of protein is sufficient to double the rate of denaturation under shaking conditions. The adverse effect of thymol can be overcome by the addition of the protectant Zonyl N (about 6 molecules Zonyl per 100 molecules of thymol). In a similar way, the protective effect of Zonyl, or of PEG 6000, can be neutralized by the addition of the anionic surfactant, aerosol OT (American Cyanamid). Here again the behavior is similar to that of these compounds on Avicelase (6, 7).

C. Proteolysis of β-Lactoglobulin in the Native (N) and in the Shake-Denatured (DN) Forms (Table III)

Two proteases were selected for these tests. One, mutanolysin, has a high optimal pH (8.+); the other, pepsin, has a low optimal pH (2.0) for activity. The substrates were incubated (unshaken) with enzyme at pH 2.0, 5.0, and 7.0. The denatured β-lactoglobulin was insoluble at pH 5.0, but soluble at pH 2.0 and 7.0. The native protein was soluble at all pHs.

At pH 5.0 the denatured protein was hydrolyzed five times as fast as the native protein—in spite of the fact that the former was insoluble, the later soluble. This is in agreement with numerous observations that denaturation of a protein increases its susceptibility to proteolysis, and indeed it is the basis for the use of increased susceptibility as an indication that a protein has been denatured (13, 16). However, the differences in rates of proteolysis (native vs DN) are much less when tested at pH 2.0 and at pH 7.0, even though the denatured protein was soluble at these pHs.

The above results are in agreement with those of shaking experiments in which protease (mutanolysin) was added to native β-lactoglobulin. The hydrolysis increased from 13% in unshaken flasks to 42% in shaken (360 rpm, 2 hr, 50°C). Polyethylene glycol (0.1 mg/ml) reduced the rate of hydrolysis in shaken flasks by virtue of its

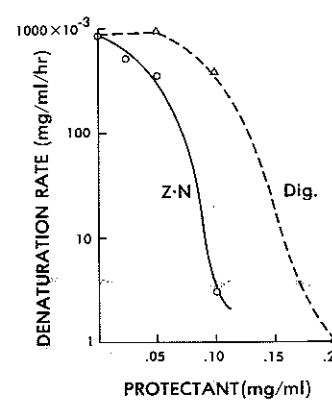

FIG. 4. Effect of concentration of "protectant" on rate of denaturation of β-lactoglobulin. *Conditions*: β-Lactoglobulin 1 mg/ml M/40 citrate buffer pH 5.0 containing protectant at various concentrations. ○——○, Zonyl N; △——△, Digitonin; 50°C, 350 rpm.

TABLE II

Compounds Which Protect Proteins against Denaturation due to Shaking: Comparison of β-Lactoglobulin and *T. reesiei* Avicelase

"Protectant"	P_{50}[a] (mg/ml) Avicelase	P_{50}[a] (mg/ml) β-Lactoglobulin
Zonyl-N (fluorinated; nonionic)	0.001	0.024
Polypropylene glycol (PPG 2700)	0.001	0.068
Polyethylene glycol (PEG 6000)	0.012	0.022
Methyl cellulose (Methocel DS 1.89)	0.10	0.04
Steviolbioside (a sophoroside)	0.10	0.09
Ustilagic acid (a cellobioside)	0.12	0.18
Sodium lauryl sulfate (anionic)	0.20	0.07
Gelatin	0.50	0.16
Lysozyme	1+	2+
Tween 80 (oleate, nonionic)	2.4	0.02

[a] P_{50} is the amount of compound required to reduce the inactivation of enzyme (or the precipitation of β-lactoglobulin) by 50%, under the conditions used (50°C, pH 5.0, 350 rpm). Arranged in order of increasing concentrations required vs Avicelase.

ability to protect the βLG against shake-denaturation.

Native Avicelase has similarly been found to be more resistant to proteolysis than that which has been denatured by heat or by acid (author's unpublished results).

D. Renaturation of Shaking-Denatured β-Lactoglobulin (Tables IV and V)

As much as 40% of the shaking-denatured β-lactoglobulin can be renatured (i.e., solubilized at pH 5.0) simply by placing the suspension in a bath at 50°C for several hours. The action is temperature dependent. It is much slower at 30 than at 50°C. Surfactants play no role in the renaturation step (Table IV).

Essentially all of the denatured protein can be redissolved at room temperature at pH 2.0 (0.01 *M* HCl or M/40 citrate) or at pH 7.0. When these solutions are readjusted to pH 5.0 the protein remains in solution indicating a change in solubility at pH 5.0 due to the pretreatments. However, it must be noted that the above experiments involved less than 2 mg β-lactoglobulin per ml. When higher concentrations were tested, the maximum solubility of the regenerated protein was found to be 3.1 mg/ml (at 25°C), a value far below that of the native protein (Table V), but far above that of the denatured protein. The denatured protein can also be redissolved in 0.01 *N* NaOH, but most of this (60%) precipitates when the solution is readjusted to pH 5.0.

There are other indications of renaturation besides the increased solubility. First is the fact that the renatured protein can again be denatured by shaking and at a rate similar to that of the native protein (Table V). And, second is the observation that the renatured form is hydrolyzed by pepsin at rates only slightly greater than that of native β-lactoglobulin rather than at the high rates at which the denatured form is hydrolyzed (Table V).

An estimate from HPLC curves (Fig. 5) indicates that about three-fourths of the renatured material has returned to the native

TABLE III

Relative Effect of Proteases on β-Lactoglobulin (N) and on β-Lactoglobulin Denatured by Shaking (DN)

Conditions	Rate of hydrolysis (mg/min/mg enzyme) Mutanolysin N	Mutanolysin DN	Pepsin N	Pepsin DN	$R\frac{DN}{N}$ Mutanolysin	$R\frac{DN}{N}$ Pepsin
pH 5.0 (50°C citrate)	16×10^{-3}	78×10^{-3}	9×10^{-3}	45×10^{-3}	5.0	5.0
pH 7.0 (50°C phosphate)	286×10^{-3}	247×10^{-3}	0.9×10^{-3}	1.5×10^{-3}	0.9	1.7
pH 2.0 (50°C HCl-citrate)	NT[a]	NT	322×10^{-3}	333×10^{-3}	NT	1.0

[a] NT, not tested.

TABLE IV

Renaturation of β-Lactoglobulin[a]

Condition	Renaturation, as percentage of denatured	
	+ Buffer (control) (%)	+ Zonyl N (0.1 mg/ml) (%)
Unshaken 21 hr 30°C	19	18
Unshaken 21 hr 50°C	38	40

[a] Samples denatured by shaking for 35 min; then add buffer or Zonyl and incubate at 30 or 50°C unshaken. Under these conditions about 50% denaturation had occurred.

shape. The peak preceding the main peak probably represents unfolded protein of larger volume than that of native β-lactoglobulin.

These results suggest that a complete reversal to the native configuration has not been obtained. This may be due to formation of some dimers or oligomers, or to a refolding to a form unlike that of the native protein.

E. Comparison of Heat-Denatured β-Lactoglobulin with Shake-Denatured β-Lactoglobulin

Heat denaturation was studied at pH 5.0 and 80°C (unshaken). Many of the compounds found effective in preventing *shaking* denaturation (Table II) were retested under unshaken conditions. None had any protective effect against *heat* denaturation. Two compounds (the quaternary ammonium, Roccal, and thymol) which accelerated the rate of denaturation by shaking also enhanced the rate of denaturation by heat. One compound, sodium lauryl sulfate, decreased *shaking* denaturation, but increased the rate of *heat* denaturation. Another, KCl, promoted shaking denaturation (at 3–74 mg/ml) while protecting slightly against heat denaturation (at 3 mg/ml).

β-Lactoglobulin denatured by heating to 95–100°C for 5 min was much less soluble in dilute acid, buffer, 6 *M* urea, and dilute alkali than the shake-denatured protein. It could not be renatured (by acid), and it was highly resistant to proteolysis. Indeed it was hydrolyzed by proteases even more slowly than native β-lactoglobulin. In this it differed greatly from the shake-denatured material:

$$\text{Rate}\ \frac{\text{DN (shake)}}{\text{DN (heat)}} = \begin{matrix} >10 \text{ for pepsin at pH 4.9} \\ >20 \text{ for mutanolysin at pH 4.9} \end{matrix}$$

DISCUSSION

Shaking denatures many proteins. β-Lactoglobulin, Avicelase (6, 7), ovalbumin (10), interferon (17), and hemoglobin (14, 15) are among those most readily affected. Our data indicate that the amount of protein precipitated per unit time is nearly independent of its concentration. Ohnishi *et al.* (15) conclude that denaturation of hemoglobin by shaking is first order. There are also differences in the effect of temperature on the rate of precipitation. Hemoglobin (14, 15) and Avicelase (6) denaturation are much faster at higher temperatures. β-Lactoglobulin denaturation is similarly increased but only at *low* shaking rates. It is unaffected by temperature (over the range 30–50°C) at *high* shaking rates.

TABLE V

Comparison of β-Lactoglobulin in the Native, Denatured, and Renatured Forms

β-Lactoglobulin	Sol. in M/40 citrate pH 5.0	Proteolysis[a] pH 4.9 ΔOD 280/hr	Denaturation[b] by shaking ΔOD/hr
Native	>50 mg/ml	0.13	1.05
Denatured (Sh.)	0.15	1.14	—
Renatured	~3.1	0.14	1.05

[a] Pepsin 0.3 mg/ml; substrate 1.33 mg/ml; 0.05 *M* citrate pH 4.9; 50°C.

[b] β-Lactoglobulin 1 mg/ml in 0.025 *M* citrate pH 5.0; 350 rpm; 30°C.

The denaturing effect of shaking can be prevented to a large extent by the addition of surfactants or of polymers, such as polyethylene glycol or methylcellulose. The amount of protectant can be as little as two parts per 100 of β-lactoglobulin (Table I), 0.2 parts per 100 of Avicelase (5). Others have reported somewhat more of Tween 80 for protection of interferon (17), and of 2,2-dimethyl propane for protection of hemoglobin (14). On the other hand, compounds like toluene and thymol *speed up* the shaking denaturation of hemoglobin (14), β-lactoglobulin, and Avicelase (6).

Our first explanation of denaturation by shaking was that it was due to shearing (9). Sedmak (17) came to a similar conclusion regarding the shaking of interferon. Indeed, when we forced our enzymes through fine tubing (9), we did observe deactivation of the sort reported by Charm (18) and Tirrell (19). We now believe that shear is a factor but a minor one in the shaking inactivation, and that air–liquid surface effects play the major role. We accept the explanation of MacRitchie (20) that protein molecules are adsorbed at the air–liquid interface, where they are unfolded, form aggregates, and are later precipitated. In the absence of shaking, a film forms that creates a barrier to diffusion of more protein molecules to the interface. On shaking, precipitated film material is removed from the interface, leaving it clean for more protein to be adsorbed, and denatured. Surfactants are similarly adsorbed and spread at the interface, but the energy barrier to their adsorption is much less than that for protein. As a result, when surfactants are added to the protein solution, they are preferentially adsorbed, occupy the surface, and by so doing prevent the unfolding and denaturation of the protein. An additional factor seems to be involved; that is, hydrophobic bonding of surfactant with protein. If only competition of surfactant and protein for the surface were involved, the order of effectiveness of surfactants (Table II) should be correlated with their surface-active property, and the same surfactant should be the most protective in *all* protein solutions. As this is not the case, hydrophobic complexing may be a factor. We suggest that the complex occupies the surface, the most hydrophobic portion (surfactant) being exposed to the gas phase, the bound protein remaining in the aqueous phase in the native conformation. On the other hand, the protein might be unfolded, but prevented from precipitating because the surfactant occupies the sites of aggregation.

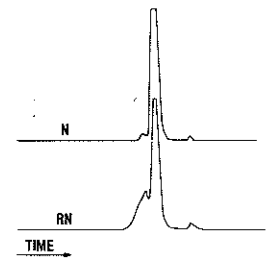

FIG. 5. Comparison of native (N) and renatured (RN) β-lactoglobulin HPLC on ^{125}I gel filtration column; eluant Tris pH 7.0. Ordinate OD 280.

Shaking denaturation of β-lactoglobulin seems to be a relatively mild change, since the product can readily be resolubilized with properties approaching those of the protein in its native conformation. This type of denaturation is easily prevented by the inclusion of small amounts of surfactant. The denaturation by heat, on the other hand, is much more extreme, more difficult to reverse, and more difficult to prevent. There are reports, however, that in some systems it may be possible to lessen the effect of heat on the conformation of the protein. Thus, Boyer *et al.* (21) some years ago found that the addition of caprylate increased the heat (and urea) stability of serum albumin. Caprylate is, of course, a surfactant. Yet in our systems such surfactants were *not* effective in preventing *heat* denaturation. The caprylate effect may, however, be specific for albumin, since it is well known that this protein has a strong affinity for fatty acids.

ACKNOWLEDGMENTS

The concepts expressed here result from many suggestions received from Drs. A. M. Klibanov, F. MacRitchie, and P. Dunn. We also thank Dr. Mary Mandels for her assistance and counsel and Waters Associates for the HPLC data (Fig. 5).

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